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14. ABSTRACT
Currently the diagnosis of prostate cancer rests on the results of a biopsy. There are over one million biopsies performed in the U.S. alone every year. Most, about 63%, are negative. Several large series have revealed that 30-40% of these miss tumors in spite of taking 12 or more cores under ultrasound guidance. Patients recommended for repeat biopsy usually in 3-12 months receive no treatment while tumor may progress. However all biopsies contain ample stroma. Others and we have observed hundreds of gene activity changes in stroma near tumor. We have developed and published a general classifier for the diagnosis of prostate cancer based on an RNA profile of 131 genes derived from analysis of frozen prostatectomy and normal stroma samples. It has been tested on 364 independent cases and shown to be 97% accurate. Preliminary studies indicate that equivalent performance may be obtained from ~45-65 genes with the highest differential expression.

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1. **INTRODUCTION:**

The year 1 progress report noted that:

Task 1, subtask 1 and **subtask 3** and **Task 2, subtasks 1** and **2** are complete or essentially complete**.**

Here we report progress on **Task 2, subtask 2** and **Task 3 subtask 3.**

2. **KEYWORDS:** Prostate cancer/formalin-fixed paraffin-embedded/tissue/diagnosis /microenvironment/stroma/validation/multigene classifier/

3. **OVERALL PROJECT SUMMARY**

Background. Conversion of biomarkers to qPCR assays on FFPE biopsies samples. As noted last year, we have previously developed a diagnostic (1) and prognostic (2) assays for prostate cancer based on analysis of Affymetrix gene expression arrays which were hybridized with RNA from fresh frozen prostate cancer tissue. Both projects utilized *tumor-adjacent stroma* or microenvironment tissue. The Diagnostic Classifier utilized tissue of known diagnosis while the Prognostic Classifier utilized tissue from prostate cancer cases with known clinical outcome of either having undergone post-surgery recurrence of cancer or were known to be recurrence-free for at least five years post-surgery. Genes selected as members of the final classifiers are based on the use of a 10-fold cross validation selection process as implemented with the program Prediction Analysis for Microarrays or PAM. The overall goal is to convert the PAM classification method to utilize qPCR values from patient biopsy tissue*, i.e.* from FFPE tissue. There are two steps, retraining PAM and validation of the qPCR based assay. Retaining comprises determining which of the genes used for frozen tissue classifier works well on FFPE RNA. In the cases of the Prognostic Classifier, many alternative genes were included in the retaining process. These genes are included in the fabrication of 384-well plate microfluidics cards for PCR of FFPE RNA. The numbers of genes on the training cards for migration to the FFPE classifiers and the number of genes selected by PAM for the current FFPE Diagnosis and Prognosis Classifiers are summarized in **Table 1.**

Table 1: Numbers of genes represented on PCR CARDS and final numbers of genes selected by 10-fold cross validation (PAM) as a classifier for prostate cancer using fresh or FFPE tissue.

FUNCTION	TRAINING CARD[1,3.] FFPE ONLY (primer sets/card)	PAM-Selected CLASSIFIER[2.]	
		FFPE	FROZEN TISSUE (probe sets)
Diagnosis	**89 (4)**	**37**	**114 (131)**
Prognosis	**186 (2)**	**14**	**15 (19)**

1."CARD" denotes a 384 well microfluidics card preprinted with primer pairs and TaqMan reagents;
2. PAM (Prediction Analysis for Microarrays), utilizes 10-fold cross validation for selection of genes from a starting set such as all the genes on the training card to derive the gene set of a classifier.
3. The training cards include additional primer pairs for 3-6 housekeeping genes with each set of experimental primer pairs.
Highlighted values are updated from the year 1 report.

3B. OVERALL PROJECT SUMMARY: NEW FOR YEAR 2.

Task 2, "Retaining: recalibrating PAM using qPCR values.
Subtask 2. "Retraining PAM".

A. Diagnosis. During year 2 we have started the retraining of our Diagnostic Classifier to use FFPE tissue. The diagnosis classifier uses stroma of negative biopsies to determine whether there is tumor present in the environment of the biopsy cores. Most biopsies in the U.S., 65%, are negative (3-5) and up to 40% of these are false negative biopsies. This is probably due to the fact that the tiny cores that are routinely collected in the clinic, for examples 12-core biopsies, only sample about 0.5% of the volume of the prostate.

Our Diagnosis Classifier is sensitive to the presence-of-tumor up to 1 mm from the biopsy core thereby greatly extending the volume of the prostate gland that is sampled. This is because there are gene expression changes in the stroma that are promoted by the nearby tumor owing to paracrine factors secreted by the tumor cells and possibly contributed to by the "cancerization field effect" (6,7),

20 FFPE prostate tissues have been used for retraining composed of 12 true positive and 8 true negative cases. 10-fold cross validation as implemented by the PAM program was used to select for the most successful genes of an input list of 89 genes that had been measured for all 20 tissues by qPCR. A FFPE Diagnosis Classifier that uses 37 genes was developed. The FFPE Diagnosis Classifier is **95%** accurate with the 20 cases of known positive or negative diagnosis. A random result is 50% accuracy. Thus this preliminary result strongly supports that FFPE RNA of stroma of prostates that do or do not harbor tumor may be used to develop an accurate FFPE Diagnosis Classifier based on stroma in agreement with our previous classifier based on Fresh frozen tissue.

This effort is continuing.

B. Prognosis. The Progress Report for Year 1 for Subtask 2 we reported the creation of an FFPE Prognosis Classifier by retaining genes originally based on the analysis of fresh frozen prostate tumor-adjacent stroma tissue (1). We used 25 cases composed of approximately equal number of recurrence and non-recurrence cases. Here is reported the extension of this work to 40 cases. The additional cases were use for the preparation of tissue sections at 20 microns thickness mounted on plastic slides, the sites of the of tumor-adjacent stroma were manually outlined on the adjacent H & E sections, then the corresponding sites of the plastic slides were removed by superimposing the plastic slides over the marked H & E sections and removing the identified tissue by punch for RNA preparation and application to our qPCR cards.

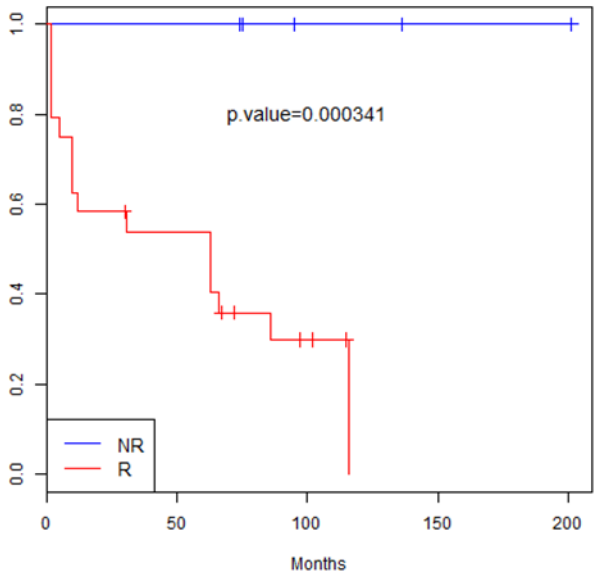

Figure 1. Kaplan-Meier curve for the classification of patients as either recurrence or non-recurrence following prostatectomy based on the expanded FFPE Prognosis Classifier trained with 40 cases.

The 40 cases consisted of (23 recurrence cases with average disease-free-survival of 35.3 months; 17 on-recurrence cases, average follow-up 82.1 months. Retaining was carried by 10-fold cross validation as implemented by the PAM Predictive Analysis for Microarrays) program. The retraining with FFPE RNA yielded a preliminary FFPE Prognosis classifier with a sensitivity of **92%,** *i.e.* 92% of the true recurrence cases were correctly identified as post-prostatectomy recurrence cases. The corresponding Kaplan-Meier curve (**Figure 1**) shows a highly significant difference (p = 0.00034) in the observed survival of the two populations identified by the preliminary FFPE Prognosis Classifier. No recurrence cases (red curve) were misclassified as non-recurrence cases (blue curve). We emphasize that these results were obtained by qPCR analysis of FFPE tissue taken from the prostatectomy tissue obtained on average 35.3 months prior to the clinical diagnosis of recurrence. Thus these results support the conclusion that the preliminary FFPE Prognosis Classifier accurately predicts recurrence years ahead of the detection of recurrence based on gene expression changes of FFPE tumor-adjacent stroma. Patients with a prognosis of recurrence following prostatectomy may be considered at "high risk" and should consider adjuvant therapy immediately following prostatectomy. This is a new clinical application.

It is recognized that this preliminary FFPE Prognosis Classifier is still suboptimal. The operating characteristic are sensitivity = 92%, specificity = 65%, and overall accuracy = 78%. The accuracy and specificity are less than those of the frozen tissue Prognosis Classifier (2). This is likely due to (i) the samples were not balanced between recurrence and non-recurrence status and utilized 6 less non-recurrence patients than recurrence patients and (ii) although 40 cases were used, the total number of recurrence or nonrelapase cases is small.

It is planned to improve the operating characteristics by increasing the number of training cases to achieve a balance of recurrence and non-recurrence cases and to continue to increase the number of cases to a number beyond which the training operating characteristics remain stable. We will then move onto to the validation phase of the FFPE Prognosis Classifier.

Task 3. "A blinded randomized preclinical validation of the new FFPE Diagnostic Classifier."

Subtask 3a. "FFPE tissue source. "

There have been significant delays in obtaining tissue for the validation of the diagnostic classifier. Tissue suitable for validation must be two kinds of biopsies: initial biopsies that were observed to be negative and subsequently found to be prostate cancer cases by a second biopsy (i.e. "true positive" cases) and initial biopsies that were observed to be negative and subsequently confirmed to be negative on one or more follow-up biopsies (i.e. "true negative " cases). Prior to the approval of this grant, we located suitable material with the Southwest Oncology Group (SWOG) which resulted from their PCPT (Prostate Cancer Prevention Trial). We applied to obtain this material as 50 true positive cases and 50 true negative cases to be provided as recuts of all biopsy cores, over 2600 slides. The application went through the SWOG formal peer-review process and was approved. This approval was part of our DOD application for the current project. In June of 2014 we renewed our request with our SWOG contact and pathologist, Dr. Scott Lucia. We further suggested that we first receive only six PCPT cases as a trial that our methods could retrieve ~90 ng RNA from each case, the required amount for assay on our microfluidic cards. Our microarray Qpcr method only requires 0.5 ng per qPCR reaction. There are 89 genes on our retraining qPCR cards for a minimum requirement of 44.5 ng or ~90 ng with a margin of safety. The extraction of 90ng of RNA from FFPE tissue is a challenge as the biopsy cores are tiny with a nominal tissue diameter of 0.98 mm in diameter by up to 2 cm long.

The six test cases were not provided until October 2014 owing to a back order delay in obtaining plastic histology slides that are required to mount the sections. Our partial analysis using our then current RNA preparation method, a Qiagen GmbH FFPE RNA preparation kit which we had optimized, was submitted to Dr. Lucia in March of 2015. We reported average total yields of **42** ng for 7 preparations using the trial material from SWOG (**Table 2**). The result was reviewed by a SWOG committee in June of 2015 and Dr. Catherine Tangen of SWOG requested that D. Mercola present an illustrated presentation of progress to the SWOG oversight committee meeting by teleconference on July 20, 2015. At that presentation D. Mercola presented further progress and improved yields. The Qiagen method was improved by substituting the protease-K reagent for protease-K from Roche Pharmaceutical Co. AG termed the Qiagen/Roche method (**Table 2**). D. Mercola suggested applying this method to the unused trial cases and reporting back.

Table 2: Summary of RNA yields from FFPE tissue by 3 methods [1.]
(see Appendix for details)

Method	Qaigen	Qiagen/Roche	Norgen
Number of preps	7	10	6
Average yield ng	42.32	72.33	160.57
Std dev	23.84	49.97	67.47
Std dev/av	0.53	0.69	0.42

of1. Note that the SWOG samples (cols. 2 & 3) were 10 micron thick tissue section while the Norgen (col. 4) samples were 20 microns and the results should be divided by 2 for comparison to the SWOG samples.

The unused cases have now been analyzed by the optimized Qiagen/Roche method which yields an average yield of **72** ng (**Table 2**) or close to the required 90 ng

Since then, we have investigated additional methods. In particular Norgen Biotek Inc. of Canada has recently offered and method with improved performance. We have used this method on our own FFPE tissues and indeed confirm significantly improved average yields of **160** ng (**Table 2**).The latter result was obtained using 20 micron thick sections compared to 10 micron thick sections provided by SWOG. Thus, the total RNA yield should be halved for comparison to the total RNA yields for the SWOG samples.

We will request the entire validation set as soon as the FFPE Diagnostic Classifier training (reviewed above in Task 2) is complete.

The details of all RNA preparations summarized in **Table 2** are included in the Appendix (**Tables A2 – A3**).

4.-KEY RESEARCH ACCOMPLISHMENTS:

- New with this year we report the development of a preliminary FFPE multigene Diagnosis Classifier with very promising operating characteristics. Such a classifier has the clinical application of being able to use RNA from patient prostate biopsies that are reported as negative for tumor and therefore composed predominately of stroma and classify the biopsy as "presence-of-tumor" or "negative" together with a probability of this determination. Presence-of-tumor would indicate that the anatomical biopsy was a false negative and that a repeat biopsy is required asap. Negative would support the anatomical biopsy.
- New with this year we report the extended number of cases applied to the training of the multigene FFPE Prognosis Classifier. The operating characteristics remain promising. The clinical application of the classifier to utilize tumor-adjacent stroma RNA of patient biopsies to determine whether prostatectomy will be followed by recurrence indicating that adjuvant therapy should be considered or whether surgery alone is likely curative. We will continue to extend the training and initiate validation.
- A major challenge of preparing sufficint RNA from patient biopsies, especially the SWOG PCPT TRIA L samples has been addressed by identifying a high yield method. However this issue of extracting sufficient RNA from the tiny fixed tissue cores of patient biopsies has led to a significant delay. In progress and the samples for validation of the FFPE Diagnosis Classifier have not yet been obtained.

5.- CONCLUSION:

The retraining of the frozen tissue Diagnosis Classifier has been initiated and the preliminary multigene FFPE Diagnosis Classifier exhibit promising operating characteristics.

The multigene FFPE Prognosis Classifier has been extended in exhibits promising operating characteristics.

The difficulty in preparing sufficient RNA from FFPR tissue from SWOG PCPT patient biopsy cores has been largely overcome however this has imposed significant delays.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

None to Report.

7. INVENTIONS, PATENTS, AND LICENSES:

None to Report.

The results of this project will support a continuation in part to a patent filing prior to this DOD project: U.S. Application Serial No. **13/857,060** for "Materials and Methods for Determining Diagnosis and Prognosis of Prostate Cancer" by The Regents of the University of California in pursuit of Dan Mercola, Michael McClelland, Zhenyu Jia,Yipeng Wang and Xin Chen. Council: Fish and Richardson P. C., reference no. 23791-0003002, April 4, 2013. Allowed March 4, 2014**.**

8. REPORTABLE OUTCOMES:

Nothing to report.

9. OTHER ACHIEVEMENTS:

The PI has served on a Department of Defense CDMRP, Prostate Cancer, review panel, Molecular Biology, TRN-CMB, H. Schwartz, SRA.

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11. APPENDICES:

Tables A1 – A3. The details of the RNA preparations summarized in **Table 1** are included in the Appendix (**Tables A1 – A3**). Table A1 is the same at **Table 1**. **Table A2** lists samples from SWOG. Yellow highlight indicates those used for RNA preparation by the "Qaigen" method. The Blue highlight indicates the SWOG samples and results for method "Qiagen/Roche". Note the increase total RNA yields. **Table A3** lists the results for .the "Norgen" method. Mauve highlight indicates the RNA concentrations and total RNA yields by the Norgen method. It is important to note that the SWOG samples were 10 micron thick tissue sections while the UCI Pathology Archive samples were studied as 20 mircon thick sections. Thus the Norgen results should be divided by two for comparison to the Qaigen and Qiagen/Roche results.

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Table A1: Summary of RNA yield by three methods

Method	Qaigen	Qiagen/Roche	Norgen
Number iof preps	7	10	6
Average yield ng	42.32	72.33	160.572
Std dev	23.843	49.967	67.467
Std dev/av	0.525081379	0.690819853	0.42016665

Table A2: RNA yields by two different methods from SWOG PCPT Trial 10 micron FFPE Biopsy tissue sections

Accession no	Location	Biopsy date	Qiagen RNA Concn. pg/µL	Qiagen Total RNA (Concn x 20 ul)	Qiagen RIN	Qaigen/Roche RNA Concn. pg/µL	Qaigen/Roche Total RNA Concn. X 20 ul)	Qaigen/Roche RIN
107-0266	R Apex	81999	754	15080	2.2			
107-0266	R Mid	81999	1295	25900	2.5			
107-0266	L Mid	81999	2705	54100	2.5			
			4754	95080				
110-0007	L-Apex	30499	2150	43000	2.6			
110-0007	L Mid	30499				1692	33840	2.5
110-0007	R Apex	30499				1719	34380	2.5
114-0036	R Mid	60700				5619	112380	2.5
114-0036	L Mid	60700				1938	38760	2.5
114-0036	L Base	60700				1935	38700	2.5
116-0097	L Base	92099				2376	47520	2.9
116-0097	R Mid	92099				4359	87180	2.5
116-0097	L Apex	92099				8793	175860	2.4
165-0094	R Base	71599	2470	49400	2.4			
165-0094	R Apex	71599				1608	32160	2.5
165-0094	L Mid	71599				6126	122520	2.5
199-0055	L Apex	10700	neg.					
199-0055	R Apex	10700	2854	57080	2.3			
199-0055	R Mid	10700	2063	41260	2.4			
AVERAGE			**2380.63**	**47612.50**	**2.41**	**3616.5**	**72330**	**2.53**
sdev			**1192.16**	**23843.00**	**0.13**	**2498**	**49967**	**0.13**
sdev/average			**0.50**	**0.50**	**0.05**	**0.69**	**0.69**	**0.05**

Table A3: RNA yields by the Norgen BIoTek kit method for UCI 20 micron FFPE prostate tissue sections

Accession n	Location	Norgen method RNA Concn. (pg/ul)	Norgen method Total RNA (Concn x 36 ul)	RIN
S03-8415	(Prostatectomy)	5063	182268	2.3
S08-1390-2	(Prostatectomy)	2586	93096	2.4
S08-1390-1	(Prostatectomy)	6021	216756	2.2
S12-9975	(Prostatectomy)	7098	255528	2.2
S-10352	(Prostatectomy)	3065	110340	2.4
S03-5358	(Prostatectomy)	2929	105444	2.4
AVERAGES		**4460.33**	**160572.00**	**2.32**
sdev		1874	67467	0.1
sdev/average		0.41	0.42	0.04
		4.5 ng/ul	160 ng	
		67467.56405	need 90 ng!	